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Proteomic analysis of a eukaryotic cilium

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Cilia and flagella are widespread cell organelles that have been highly conserved throughout evolution and play important roles in motility, sensory perception, and the life cycles of eukaryotes ranging from protists to humans. Despite the ubiquity and importance of these organelles, their composition is not well known. Here we use mass spectrometry to identify proteins in purified flagella from the green alga *Chlamydomonas reinhardtii*. 360 proteins were identified with high confidence, and 292 more with moderate confidence. 97 out of 101 previously known flagellar

proteins were found, indicating that this is a very complete dataset. The flagellar proteome is rich in motor and signal transduction components, and contains numerous proteins with homologues associated with diseases such as cystic kidney disease, male sterility, and hydrocephalus in humans and model vertebrates. The flagellum also contains many proteins that are conserved in humans but have not been previously characterized in any organism. The results indicate that flagella are far more complex than previously estimated.

Introduction

Defects in cilia in humans have long been known to cause the severe inherited disorder primary ciliary dyskinesia; more recent evidence has implicated cilia in polycystic kidney disease (PKD), retinal degeneration, obesity syndromes, and developmental disorders (Pazour and Rosenbaum, 2002; Ong and Wheatley, 2003; Scholey, 2003; Snell et al., 2004; Pazour, 2004). Cilia and flagella, which are essentially identical organelles and hereafter referred to interchangeably, also are essential to the life cycles of many human and animal parasites, including those that cause malaria, African sleeping sickness, Chagas' disease, and giardiasis. Some cilia, such as airway cilia and sperm tails, move; others are nonmotile and serve as sensory antennae (Pazour and Witman, 2003). Structurally, the cilium (Fig. 1) consists of a microtubular axoneme ensheathed by a ciliary membrane that is continuous with the plasma membrane of the cell. The axoneme of motile cilia includes substructures such as the dynein arms and radial spokes that generate and control axonemal bending. The cilium also contains "matrix" proteins that are not tightly associated with either the membrane or the axoneme, including proteins involved in

intraflagellar transport (IFT), a process required for assembly and maintenance of the organelle (Rosenbaum and Witman, 2002).

An understanding of how cilia carry out their functions and of their role in disease will require increased knowledge of their protein composition. Previous two-dimensional gel electrophoretic analyses of isolated flagella from the green alga *Chlamydomonas reinhardtii*, a genetically tractable organism that has many advantages for biochemical and molecular genetic studies of the flagellum, revealed that flagella contain more than 250 proteins (Luck and Piperno, 1989; Dutcher, 1995), of which only ~100 have been characterized at the molecular level. Recently, the availability of complete genome sequences has made possible global approaches to the identification of genes encoding ciliary and flagellar proteins. For example, by comparing the genomes of ciliated versus non-ciliated organisms, Avidor-Reiss et al. (2004) and Li et al. (2004) identified 187 and 688 genes, respectively, that are present in the former but not the latter; these are candidates for encoding flagellar and basal body components. In a different approach, Stolc et al. (2005) used a gene chip to examine the level of induction of all *C. reinhardtii* genes after deflagellation; they identified 220 genes that were induced at least 100% and therefore likely to be involved in the assembly or function of ciliary or flagellar proteins.

Although the above approaches can provide very valuable information on proteins associated with cilia and basal bodies, they are a complement to and not a substitute for direct

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Abbreviations used: FAP, flagellar associated protein; IFT, intraflagellar transport; InsP3, inositol 1,4,5-trisphosphate; MS, mass spectrometry; PKD, polycystic kidney disease; PMCA, plasma membrane Ca^{2+} ATPase.

The online version of this article contains supplemental material.

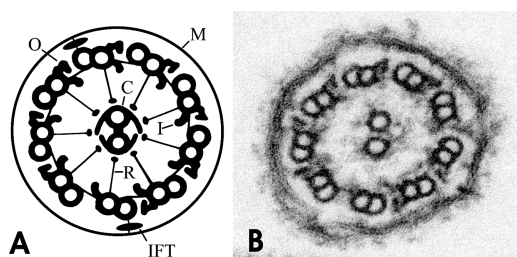


Figure 1. **Flagellar structures.** Diagram (A) and electron micrograph (B) of a cross section of a motile flagellum (from *C. reinhardtii*). (C) Central pair of microtubules; (I) inner dynein arm; (IFT) IFT particle; (M) flagellar membrane; (O) outer dynein arm; (R) radial spoke.

proteomic analyses using mass spectrometry (MS). For example, comparative genomic approaches cannot readily identify genes encoding flagellar proteins, such as kinesins and many signal transduction proteins, that have close homologues in plants, and examination of gene induction during flagellar regeneration is likely to miss many proteins that function in both the flagellum and cytoplasm. In contrast, such proteins can be readily identified by a proteomics approach, which also can uniquely provide an indication of the abundance of a protein and its distribution in the flagellum. A preliminary proteomic analysis of detergent-extracted ciliary axonemes from cultured human bronchial epithelial cells identified 214 proteins (Ostrowski et al., 2002); however, this study was compromised by the presence of other cellular structures in the axonemal preparation, and by limitations in the amount of material available and/or sequence data obtained, with the result that only 89 of the proteins were identified by more than a single peptide. Here, we use MS to identify the proteins in biochemically fractionated *C. reinhardtii* flagella, which are available in large amounts and in high purity.

Results

Identification of flagellar proteins

To identify the proteins that compose *C. reinhardtii* flagella, these organelles were released from vegetative cells by dibucaine treatment, isolated from the cell bodies by low speed centrifugation and sucrose step gradient fractionation, and harvested by high-speed centrifugation. The membranes of flagella isolated in this way generally remain intact and the matrix remains in situ (Fig. 2 A). The purified flagella were then fractionated into a Tergitol-insoluble fraction containing membrane and axonemes (Fig. 2 B), or into a Nonidet-soluble fraction containing membrane + matrix proteins, a fraction containing proteins released from the Nonidet-demembranated axonemes by KCl extraction, and a fraction containing the axonemal proteins remaining after KCl extraction (Fig. 2 D). Electron microscopy of isolated flagella and axonemal fractions indicates that they are highly pure (Fig. 2).

Initial analysis was performed on the Tergitol-insoluble fraction isolated from wild-type flagella. However, peptides derived from the outer dynein arm were very abundant, and concerns that these might prevent identification of peptides

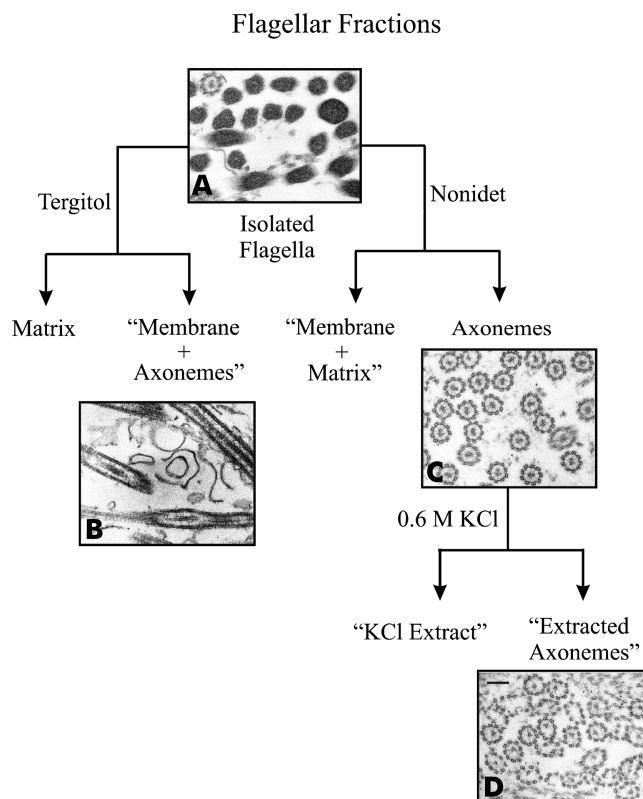


Figure 2. **Flow chart for isolation of flagellar fractions used for MS analyses.** (A) Electron micrograph of cross sections of isolated flagella. Most of the flagella have an intact membrane; in these flagella, the matrix is dense and obscures the axonemal microtubules. In a few of the flagella, the membrane has ruptured, releasing the matrix into solution and revealing the microtubules. No other cell organelles are apparent in the flagellar preparation. In the initial analysis (left pathway), isolated flagella from wild-type cells were treated with the detergent Tergitol, which disrupts the flagellar membranes without dissolving them, and releases the flagellar matrix. The Tergitol-insoluble fraction (B) was then collected by centrifugation and analyzed by MS as described in the Materials and methods. For subsequent analyses (right pathway), flagella were isolated from the outer armless mutant *oda1* and treated with the detergent Nonidet, which dissolves the membrane and releases the matrix; the preparation was then centrifuged to yield a supernatant containing the "membrane + matrix" fraction and a pellet containing the demembranated axonemes (C). The axonemes were resuspended in 0.6 M KCl and the mixture was centrifuged to yield a supernatant containing the "KCl extract" and a pellet containing the "extracted axonemes" (D). The KCl extraction releases numerous axonemal proteins, including those of the inner dynein arms and the C2 central microtubule, which are missing in the extracted axonemes. The "membrane + matrix," "KCl extract," and "extracted axonemes" were then analyzed by MS. Bar, 0.2 μ m. (B) Micrograph courtesy of M. Wirschell (Emory University, Atlanta, GA).

from less abundant proteins prompted us to use flagellar fractions isolated from an outer dynein arm mutant (*oda1-1*) for the remaining work. The proteins in each of the four fractions were separated by one-dimensional SDS-PAGE, each gel lane cut into 33 to 45 slices (Fig. S1), the proteins in each slice digested with trypsin, and the resulting peptides eluted, separated by HPLC, and analyzed by MS-MS using electrospray ionization and an LCQ ion trap mass spectrometer. The search engine Mascot was used to find the best matches to the MS-MS spectra in the translated *C. reinhardtii* genome, and the peptides also were searched against a database containing all predicted *C. reinhardtii* proteins to identify the origins of those that

Table I. Summary of results

Proteins identified by five or more peptides	360
Proteins identified by two to four peptides	292
Proteins identified by one peptide	482
Major groups ^a	
Signal transduction proteins	93
Motor proteins	41
Nucleotide metabolism	9
Glycolytic enzymes + malate dehydrogenase	8
Conserved uncharacterized proteins	87
Predicted membrane proteins	39

^aOnly proteins identified by two or more peptides are included here. Proteins may be included in more than one category.

spanned exon–exon junctions. From these four fractions, 8,345 unique peptides were identified and grouped by the proteins from which they were derived. 360 proteins were identified by five or more unique peptides, 292 by two to four unique peptides (Table S1) and another 482 by a single peptide (Table S2). All protein and peptide sequences, as well as the Mascot scores for the peptides, are available at <http://labs.umassmed.edu/chlamyfp/index.php>.

The list of flagellar proteins identified by two or more peptides is rich in motor proteins, signal transduction proteins, proteins with predicted coiled-coil domains, and predicted membrane proteins (Table I), and contains a number of proteins whose homologues are associated with disease in humans and model vertebrates. Nearly 90 proteins are highly conserved in humans (BLAST E score $\leq 1e-10$) but have not been previously characterized in any organism. Individual proteins of interest are described in the Discussion.

The dataset contains nearly all known flagellar proteins

A measure of the completeness of the dataset can be obtained by determining what percentage of known proteins is present. At this time, 101 *C. reinhardtii* flagellar proteins have been identified by biochemical, genetic, and bioinformatic methods (Table S3). 97 of these are present in our dataset. This includes all known outer dynein arm subunits and associated proteins except one, all known and predicted inner dynein arm subunits except one, all known IFT components, and all known radial spoke proteins (Table S3). The gene (*ODA5*) encoding the outer dynein arm-associated protein that is missing is not present in the genome assembly and so could not have been found by our analysis. The gene (*DHC3*) encoding the putative inner dynein arm subunit that is missing was identified by PCR analysis of genomic DNA but the protein has not been shown biochemically to be in flagella. This gene was much less strongly expressed than other dynein heavy chain genes as measured by Northern blot or reverse-transcriptase PCR analysis (Porter et al., 1996). The gene encoding LF4 is mostly missing from the genome assembly. The only other missing protein is p80 katanin, which was shown genetically to be involved in central pair assembly, but the abundance of the native protein is unknown (Dymek et al., 2004). Although fractions from flagella lacking outer dynein arms were used for all but the initial analysis, all known outer

arm subunits were detected in that initial sample, so any previously unidentified outer arm subunits of similar abundance also are likely to have been detected. Thus, this dataset is likely to contain nearly all flagellar proteins.

Assessment of exclusiveness of the dataset

Even though electron microscopy indicated that the flagellar fractions were highly pure, at some level the dataset is likely to contain proteins that are contaminants. The number of peptides found by electrospray MS of complex mixtures roughly correlates with the abundance and size of the proteins from which they are derived (Washburn et al., 2001). Most known flagellar proteins were identified by more than five peptides; notable exceptions include several outer dynein arm subunits, which would not have been present in flagella of the outer dynein armless mutant used for all but the Tergitol-insoluble membrane + axonemes fraction; Tctex1 (one peptide), which is very small; and EB1 (two peptides), which is located only at the tip of the flagellum and therefore is of relatively low abundance. Obvious contaminants in the dataset (tRNA synthetases, ribosomal proteins, and histones) were usually identified by one or two peptides, even though these proteins are highly abundant in the cell body. Thus, the 360 proteins identified by five or more peptides are highly likely to be true flagellar proteins, with possible exceptions including methionine synthase, elongation factors, arginyl-tRNA synthetase, carbonic anhydrase, and two cell wall proteins. However, even some of the latter proteins may function in the flagellum: methionine synthase gene expression is induced by flagellar adhesion (Kurvari et al., 1995); elongation factor 1a binds calmodulin and localizes to *Tetrahymena* cilia (Ueno et al., 2003); and arginyl-tRNA synthetase is reported to modify protein substrates to allow degradation via the ubiquitin pathway (Ferber and Ciechanover, 1987), several other components of which were found in the flagellum. Several glycolytic enzymes also are present in this data subset and are likely to function in the flagellum (see Discussion).

The 292 proteins identified by two to four peptides should be considered candidate flagellar proteins as this group contains both known flagellar proteins and likely contaminants. Within this group, the genes of 19 out of 51 (37%) randomly chosen proteins that were not previously reported to be in the flagellum were found to be induced at least 1× by deflagellation (i.e., their transcript levels doubled; see Induction of genes by deflagellation section). Flagellar proteins would include these plus known flagellar proteins plus some whose genes are not induced by deflagellation, so that the percent of true flagellar proteins in this subset is likely to be substantially higher than 37%. The group of proteins identified by single peptides was not analyzed in detail but likely contains low abundance and small flagellar proteins along with contaminants.

Distribution of proteins in flagellar fractions

In the subset of proteins identified by five or more peptides, the peptides of ~3/4 of the proteins were present predominantly in the KCl extract and extracted axoneme fractions and thus

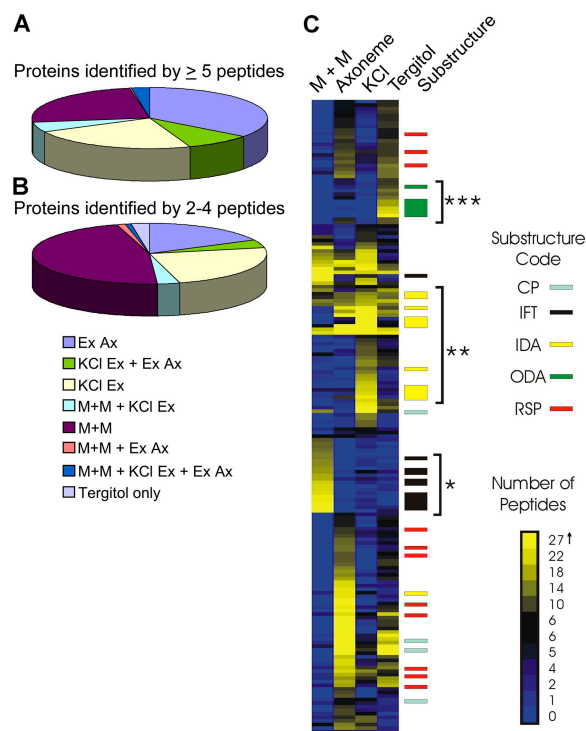


Figure 3. Distribution of proteins in flagellar fractions. (A and B) Distribution of proteins identified by five or more peptides (A) and by two to four peptides (B) in the extracted axoneme (Ex Ax), KCl extract (KCl Ex), and membrane + matrix (M+M) fractions. A protein was assigned to a specific fraction if it was identified by at least twice as many peptides in that fraction as in any other fraction. If the number of peptides in a second or third fraction was more than half that of the first fraction, the protein was assigned to two or all three fractions, as appropriate. Outer dynein arm subunits found exclusively in the Tergitol-insoluble fraction were automatically assigned to the KCl extract fraction. Seven additional proteins were identified by two to four peptides in the Tergitol-insoluble fraction only; these are included in B. (C) Hierarchical cluster diagram. Proteins were clustered based on the distribution of peptides in all four biochemical fractions. Each horizontal row represents one protein. The number of peptides in the particular fraction is represented by a color ranging from yellow (>27 peptides) to blue (0 peptides). In addition, the major known flagellar proteins are color coded under "substructure" with central pair (CP) proteins being marked in blue gray, intraflagellar transport (IFT) proteins in black, inner dynein arm (IDA) proteins in yellow, outer dynein arm (ODA) proteins in green and radial spoke proteins (RSP) in red. Note, for example, the IFT protein cluster (*) where nearly all peptides were found in the M+M fraction, the inner dynein arm cluster (**) where most peptides were most abundant in the KCl extract, and the outer dynein arm cluster (***) where nearly all peptides were found in the Tergitol-insoluble fraction. Only proteins represented by 15 or more peptides were included in the cluster analysis. A detailed version of this figure including gene IDs is available at <http://labs.umassmed.edu/chlamyfp/index.php>.

probably represent axonemal components (Fig. 3 A). As expected, this includes the previously identified inner dynein arm proteins, central pair proteins, and radial spoke proteins. The peptides of $\sim 1/4$ of the proteins were abundant predominantly in the membrane + matrix fraction; this included all proteins known to be subunits of IFT motors or particles. Within the subset of proteins identified by two to four peptides, the proteins were approximately equally distributed between the membrane + matrix and axonemal fractions (Fig. 3 B). Therefore, the majority of proteins found in the flagellar proteome are in the axonemal fractions. The difference in the distribution

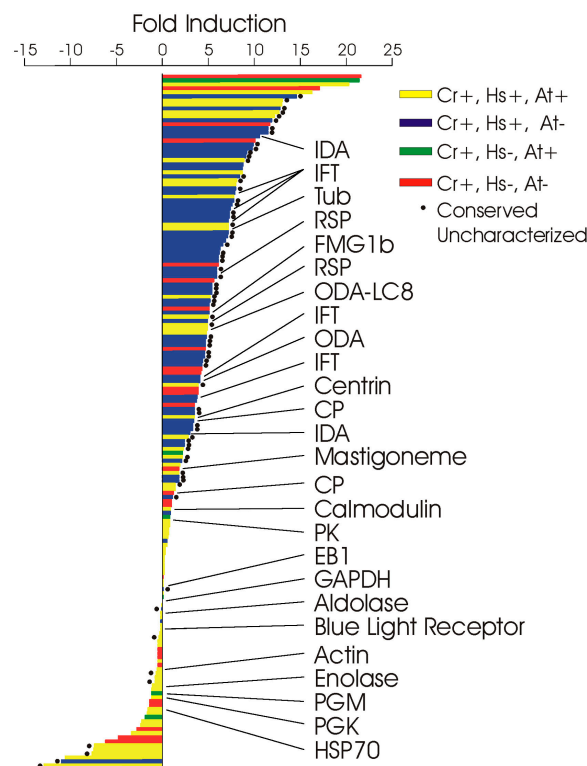


Figure 4. Deflagellation-induced change in transcript levels for 176 predicted flagellar proteins as measured by real-time PCR. (Yellow) Proteins conserved in *C. reinhardtii*, humans, and *A. thaliana* (BLAST $E \leq 1e-10$); (blue) proteins conserved in *C. reinhardtii* and humans but not *A. thaliana*; (green) *C. reinhardtii* proteins conserved in *A. thaliana* but not humans; (red) *C. reinhardtii* proteins not conserved in humans or *A. thaliana*. Black dots indicate conserved, uncharacterized proteins. 26 proteins are identified by name or structure. (CP) Central pair protein; (FMG) flagellar membrane glycoprotein; (GAPDH) glyceraldehyde 3-phosphate dehydrogenase; (HSP70) heat-shock protein 70; (IDA) inner dynein arm protein; (IFT) intraflagellar transport protein; (ODA) outer dynein arm protein; (ODA-LC8) dynein light chain 8; (RSP) radial spoke protein; (TUB) tubulin.

of proteins in the two data subsets suggests that the membrane + matrix fraction contains a larger proportion of low abundance proteins than the axonemal fractions. Fig. 3 C shows a hierarchical cluster diagram based on the number and distribution of peptides in the different fractions for proteins identified by 15 or more peptides. Proteins known to be associated with IFT, the outer dynein arm, or the inner dynein arm grouped closely together, indicating that the analysis is likely to have predictive value. Thus, novel proteins that group with these proteins may be new constituents of these structures. Proteins of previously unidentified structures or processes may similarly group with each other.

Induction of genes by deflagellation

Genes encoding proteins that are used exclusively in the flagellum are strongly induced by deflagellation in *C. reinhardtii*, whereas genes encoding nonflagellar proteins are not induced (Lefebvre and Rosenbaum, 1986). Proteins such as actin and calmodulin that function in both the flagellum and the cell body usually fall into the latter category or are only weakly induced. Therefore, to obtain a rough estimate of the percent of

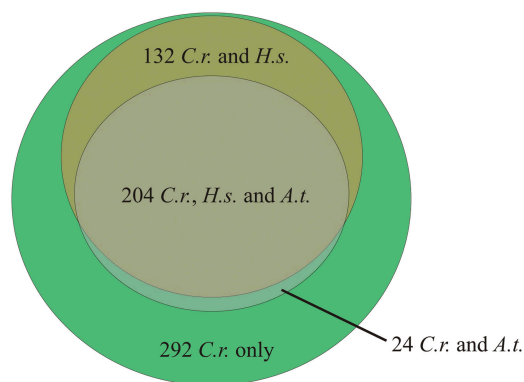


Figure 5. **Conservation of proteins in flagellar proteome.** Number of *C. reinhardtii* (C.r.) flagellar proteins conserved (BLAST $E \leq 1e-10$) in *H. sapiens* (H.s.) and/or *A. thaliana* (A.t.), out of a total of 652 proteins identified by two or more peptide hits. 292 *C. reinhardtii* flagellar proteins had no close homologues in either humans or *A. thaliana*.

the conserved, uncharacterized proteins that function specifically in the flagellum, as well as to investigate if other proteins of interest are likely to be specific to the flagellum, we used real-time PCR to measure changes in transcript level during flagellar regeneration for 176 proteins of the flagellar proteome, including 69 of the conserved, uncharacterized proteins (Fig. 4 and Table S1). Values were normalized relative to those for the G β subunit. The fold induction for known proteins believed to function specifically in the flagellum ranged from 1.8 \times for the mastigoneme protein to 11 \times for the inner dynein arm subunit Tctex2b. 60 out of 69 (87%) of the conserved uncharacterized proteins tested were induced at least 1 \times . These results indicate that a large majority of the conserved, previously uncharacterized proteins identified in the flagellar proteome function specifically in the flagellum. In addition, large inductions were observed for genes encoding several proteins of particular interest, including the hydinn homologue (7 \times), the inositol 1,4,5-trisphosphate (InsP3) receptor (6 \times), the NEK1-like NIMA kinase (13 \times), the Pacrg protein homologue (9 \times), and the plasma membrane Ca²⁺ ATPase (PMCA) C_370080 (5 \times), indicating that they also are likely to function specifically in the flagellum. Transcripts encoding enzymes of the glycolytic pathway showed little change or decreased slightly following deflagellation, as expected for proteins that function in both the flagellum and cytoplasm.

Conservation of flagellar proteins in humans and *Arabidopsis*

As noted above, comparisons of genomes of organisms such as *C. reinhardtii* and humans that have cilia with those of organisms such as *Arabidopsis thaliana* that do not have cilia have resulted in the identification of new genes necessary for cilia and basal body development and function (Avidor-Reiss et al., 2004; Li et al., 2004). The *C. reinhardtii* proteins identified in this work were analyzed by BLAST (using a BLAST E score of $\leq 1e-10$ as the cutoff) to identify similar proteins encoded by the human and *A. thaliana* genomes (Fig. 5). 336 (52%) of the proteins identified by two or more unique peptides match proteins in humans, indicating that the majority of ciliary proteins

have been conserved between *C. reinhardtii* and humans; of these, 209 identify the same *C. reinhardtii* protein in a reciprocal BLAST search, providing additional support that the two proteins are orthologous. 132 proteins (20%) are conserved in humans but not in *A. thaliana*; these would have been identified by comparative genomics approaches. However, 204 (31%) of the proteins are conserved in both humans and *A. thaliana*. These proteins include the kinesins, most of the signal transduction proteins, the enzymes involved in nucleotide metabolism, and other proteins that also function in the cytoplasm or have close family members that function in the cytoplasm. Importantly, this large class would not have been readily detected by a comparative genomics approach. 24 (<4%) of the proteins are conserved in *A. thaliana* but not humans. Some of these are likely contaminants in the flagellar preparation (e.g., Rubisco and chlorophyll-a/b binding protein, identified by two peptides each), but two out of six tested were induced more than 1 \times by deflagellation (Fig. 4), indicating that some are true flagellar proteins. These may be plant-specific proteins that have been adapted by *Chlamydomonas* to serve flagellar functions.

Discussion

In this work, we used MS to characterize the *C. reinhardtii* flagellar proteome. We identified 360 proteins by five or more peptides. Because the preparation was highly pure as assessed by electron microscopy and this group of proteins contains very few obvious contaminants, most of these are likely to be true flagellar proteins. We also identified 292 proteins by two to four peptides; we consider these to be candidate flagellar proteins that need to be confirmed by further analysis. The best previous estimate of the number of proteins in the *C. reinhardtii* flagellum was ~ 250 based on two-dimensional gels (Luck and Piperno, 1989; Dutcher, 1995). Our data indicate that the number is substantially higher and that the complexity of the flagellum is greater than previously anticipated.

Motor and signal transduction proteins

In addition to the very large number of known dynein subunits and three previously known kinesin subunits, a previously uncharacterized kinesin heavy chain (C_70192) was identified. Phylogenetic analysis did not support definitive assignment of this protein to a specific kinesin family. Peptides from this protein were found primarily in the axonemal fractions and cluster analysis (Fig. 3 C) grouped it with radial spoke and central pair components. This may be the unidentified flagellar kinesin detected by immunological methods (Fox et al., 1994). No myosin heavy chains were found.

The flagellar proteome contains over 90 putative signal transduction proteins. Based on Interpro domain analyses and homology searches, the proteins identified by two or more peptides include 21 protein kinases and 11 protein phosphatases, the vast majority of which have not previously been reported to be in the flagellum. These findings are consistent with the fact that a large number of flagellar proteins are phosphorylated (Piperno et al., 1981), and with the roles of protein phosphory-

lation and dephosphorylation in the control of flagellar motility (Porter and Sale, 2000), signaling (Pan and Snell, 2000), and probably assembly (Rosenbaum and Witman, 2002). We also found 27 EF hand-containing proteins that are potential Ca^{2+} -binding proteins, consistent with the well-established role of Ca^{2+} in the control of flagellar behavior (Witman, 1993) and signaling (Bloodgood, 1992; Praetorius and Spring, 2001). 10 proteins are predicted to have IQ (calmodulin-binding) domains. An InsP3 receptor was identified by 33 unique peptides in the membrane + matrix fraction, suggesting that it is a very abundant flagellar component. 10 small GTPases were identified, including five RAB subfamily members, four ARF subfamily members, and RAN. A RAB GDP-dissociation inhibitor that controls the GDP-GTP exchange reaction also is present. Two predicted 14-3-3 proteins, which mediate signal transduction by binding to phosphoserine-containing proteins, were identified. Six ion pumps or channels, including a homologue of human polycystin 2 (see Homologues of vertebrate disease proteins) and three predicted PMCA (C_370080, C_550076, C_700061), were present. The proteome also contained a group of four closely related proteins (all encoded by scaffold 152) that are composed of 8 to 12 transmembrane helices and a PAS domain; PAS domains are thought to be sensory motifs that are involved in detecting diverse stimuli ranging from light or oxygen to redox state and small ligands (Taylor and Zhulin, 1999). We also confirmed the presence of a second type of PAS domain-containing protein, the blue-light receptor phototropin (Huang et al., 2004), in the axonemal fraction. The identification of so many signal transduction proteins underscores the importance of signaling in the function of motile as well as nonmotile cilia, and should greatly facilitate studies to understand signaling mechanisms in these organelles (Pazour and Witman, 2003).

Nucleotide production and metabolism

Flagellar motility, assembly and signal transduction require large amounts of ATP and GTP, and the flagellum contains numerous proteins that are likely to be involved in maintaining adequate levels of these nucleotides. Two adenylate kinases and a nucleoside diphosphate kinase have been characterized in the past year (Patel-King et al., 2004; Wirschell et al., 2004; Zhang and Mitchell, 2004). We found two additional nucleoside diphosphate kinases (C_1090038, C_1230002), two additional adenylate kinases (C_750038, C_10320001), and two guanylate kinases (C_160167, C_3300001), four of which are associated with the axoneme.

Interestingly, the flagellar proteome also contains all enzymes of the late glycolytic pathway required for the conversion of fructose 1,6-bisphosphate to pyruvate, which could generate ATP for use in the flagellum (Fig. 6). Consistent with glycolysis occurring in the flagellum, we also found two isoforms of malate dehydrogenase, which could be involved in a malate-oxaloacetate shuttle to regenerate the NAD consumed by the glycolytic pathway. It might be argued that, because the early steps of the glycolytic pathway are reported to occur primarily in the chloroplast and the later steps in the cytoplasm in *C. reinhardtii* (Klein, 1986), the cytoplasmic

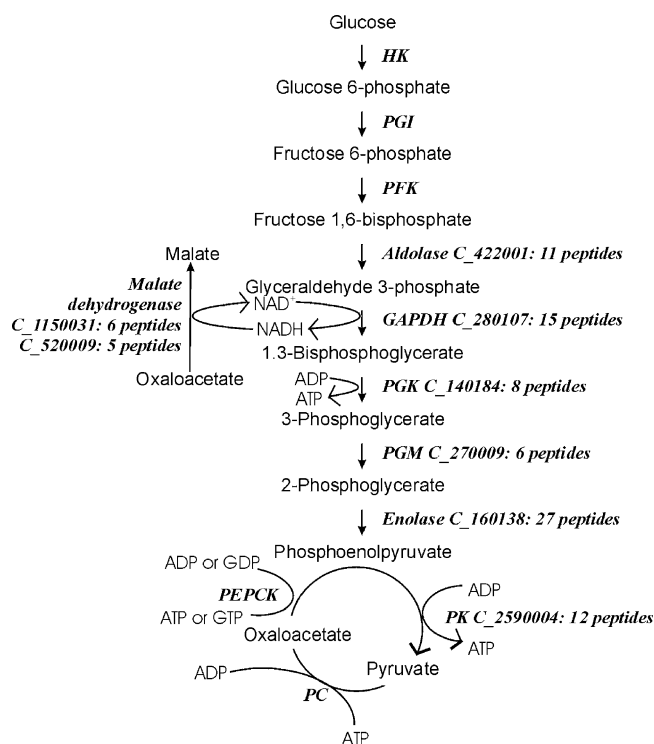


Figure 6. **Enzymes of the glycolytic pathway found in the flagellar proteome.** The number of peptides found for each protein is indicated. (HK) Hexokinase; (PGI) phosphoglucose isomerase; (PFK) phosphofructokinase; (GAPDH) glyceraldehyde 3-phosphate dehydrogenase; (PGK) phosphoglycerate kinase; (PGM) phosphoglycerate mutase; (PK) pyruvate kinase; (PC) pyruvate carboxylase; (PEPCK) phosphoenolpyruvate carboxykinase.

enzymes might simply have diffused into the flagella but are not flagellar proteins per se. Our data argue against this, because aldolase, also reported to be located primarily in the chloroplast (Klein, 1986), was identified by numerous peptides (Fig. 6). We also did not find phosphoenolpyruvate carboxylase, a cytoplasmic marker (Klein, 1986), or any enzymes of pyrimidine biosynthesis, which should be abundant in the cytoplasm. Moreover, peptides derived from glyceraldehyde-3-phosphate dehydrogenase were found almost exclusively in the axonemal fractions, indicating that this enzyme is anchored to the axoneme, and peptides derived from enolase were found in both membrane + matrix and axonemal fractions, indicating that some of the flagellar enolase is similarly assembled into the axoneme. These results suggest that there is a mechanism to exclude soluble cytoplasmic proteins from the flagellum, and that the glycolytic enzymes are true flagellar components. The localization of glycolytic enzymes to cilia may be a way to maintain a constant ATP/ADP ratio along the length of the cilium. Dynein activity is sensitive to ATP/ADP ratios (Yagi, 2000; unpublished data). If ATP were available to the cilium only via diffusion from the cell body, the ATP/ADP ratios would vary along the length of the organelle, and dynein function would be compromised. The presence of glycolytic enzymes in cilia is likely to be widespread; glyceraldehyde-3-phosphate dehydrogenase is a component of the mammalian sperm flagellum and is essential for

sperm motility and fertilization (Miki et al., 2004), and photoreceptor rod outer segments, which are developmentally derived from cilia, are reported to contain the entire glycolytic pathway as a way of maintaining the high levels of ATP needed for the formation of cGMP in this compartment (Hsu and Molday, 1994).

Homologues of vertebrate disease proteins

Cilia have been implicated in several human disorders, including PKD (Pazour, 2004). Interestingly, the *C. reinhardtii* flagellar proteome contains a homologue (C_590099) of polycystin 2, a Ca^{2+} channel defective in some cases of human autosomal PKD, and a homologue (C_420012) of fibrocystin, a protein of unknown function that is defective in nearly all cases of human autosomal recessive PKD; both of these proteins are associated with cilia in mammals. We also found homologues (C_1780022 and C_10017, respectively) of scorpion and qilin, defects in which cause cystic kidney disease in zebrafish (Sun et al., 2004). Scorpion is a small GTPase in the ARF subfamily and is necessary for ciliary assembly, whereas qilin is a coiled-coil protein, but nothing more is known about these proteins. Defects in the NIMA kinases NEK1 and NEK8 result in cystic kidney disease in the mouse (Upadhyaya et al., 2000; Liu et al., 2002); *C. reinhardtii* expresses seven NIMA kinases (Bradley et al., 2004), of which three, Fa2p (C_100034), CNK2 (C_1220044), and CNK6 (C_160119) are found in the proteome. Thus, *C. reinhardtii* should be a useful model for learning more about the functions of these important proteins and their roles in disease.

The flagellar proteome also contains homologues (C_410060 and C_610051) of hydin and napa, which are respectively mutated in the *hy3* and *hyh* mouse models for hydrocephalus (Davy and Robinson, 2003; Chae et al., 2004; Hong et al., 2004); the hydin gene also is a strong candidate

for causing hydrocephalus in humans (Davy and Robinson, 2003). Hydrocephalus is the abnormal accumulation of cerebral spinal fluid in the ventricles of the brain. The fact that hydin and napa homologues are both present in the *C. reinhardtii* flagellar proteome indicates that cilia probably are involved in the development of hydrocephalus caused by defects in these proteins.

Homologues of a *C. reinhardtii* inner dynein arm heavy chain and a central pair protein previously were shown to cause male sterility when defective in the mouse (Neesen et al., 2001; Sapiro et al., 2002). The *C. reinhardtii* flagellar proteome contains homologues of additional proteins associated with male sterility in the mouse. These include C_20334, a homologue of the Pacrg (Parkin-coregulated gene) protein (Lorenzetti et al., 2004), and C_2260011, a homologue of *Pp1cc* encoding a protein phosphatase 1 catalytic subunit γ (Varmuza et al., 1999). In addition, C_1620015 and C_10830001 are homologues of an Arf-like protein encoded by *Arl4*, which results in reduced sperm count when defective in the mouse (Schurmann et al., 2002), and C_60170 is a close homologue of a protein kinase encoded by *Mak*, which causes reductions in litter size and sperm motility when missing (Shinkai et al., 2002). The fact that homologues of these proteins are in the *C. reinhardtii* flagellum indicates that the proteins are likely to exert their effects as components of the mammalian sperm flagellum.

All three of the PMCAs in the *C. reinhardtii* flagellar proteome are closely related to the product of the mouse wriggle/deafwaddler gene, which causes deafness and balance problems when defective. This raises the intriguing possibility that the mammalian wriggle/deafwaddler gene product may function in the kinocilium of the inner ear.

Ciliary targeting sequences

Myristoylation of NH_2 -terminal glycine residues plays a role in directing proteins without membrane spanning domains to the ciliary membrane in *Leishmania* and *Trypanosoma* (Godsel and

Table II. Predicted myristoylated proteins in the ciliary proteome

Cr Model	Peptide distribution				CrFP description
	M + M	Axo	KCl	Terg	
C_10830001	4	0	1	0	ARFA1a, ADP-ribosylation factor 1
C_1620015	2	0	0	0	ARFA1b, ADP-ribosylation factor 1
C_1120033	2	0	0	0	Similar to protein tyrosine phosphatase
C_1390004	14	0	0	0	PP2C, similar to protein phosphatase 2C
C_520010	2	0	0	0	Similar to protein phosphatase 2C
C_60149	16	11	23	3	Cyclic nucleotide-dependent protein kinase
C_50062	9	0	0	0	PKG, similar to cGMP-dependent protein kinase
C_740056	3	0	0	0	Similar to cNMP-dependent protein kinase
C_1390012	15	14	19	20	ODA5-associated adenylate kinase
C_1230005	4	0	0	0	Similar to calcium-binding protein
C_1150014	3	0	1	4	Similar to carbonic anhydrase
C_700061	0	0	2	3	Similar to calcium-transporting ATPases
C_310066	3	0	0	0	Weak similarity to elongation factor 1 γ
C_780027	2	0	0	0	Sugar phosphatase domain protein
C_850051	10	0	0	0	FAP138, flagellar-associated coiled-coil protein
C_80226	16	4	11	18	FAP12, uncharacterized protein with lipase domain
C_90072	2	0	0	0	Hypothetical protein
C_1080051	2	0	0	1	Hypothetical protein

Engman, 1999; Tull et al., 2004). To determine if the myristoylation motif was enriched in ciliary proteins, all 19,832 predicted *C. reinhardtii* proteins were analyzed using a myristoylation prediction algorithm (Maurer-Stroh et al., 2004). The program predicted that 103 proteins (0.5%) were myristoylated. Of these, 18, representing 2.7% of all flagellar proteins, were found in the flagellar proteome. Thus, proteins predicted to be myristoylated are enriched in the flagellum. These proteins were found primarily in the membrane + matrix fraction and represented 8% of all such proteins (Table II). Interestingly, this motif may also function in ciliary targeting in mammals because cystin, the gene product of the mouse cystic kidney disease gene *Cpk*, also contains this motif and is localized to cilia (Hou et al., 2002). A number of the mammalian homologues of the potentially myristoylated *C. reinhardtii* proteins also contain the myristoylation motif. For example, human ARF4, 5, and 6 are predicted to be likely myristoylated whereas several of the others are predicted to be potentially myristoylated. Mammalian protein phosphatase 2 γ (but not the other protein phosphatase 2c isoforms) is predicted to be myristoylated as is mammalian type II cGMP-dependent protein kinase (but not type I), suggesting that protein phosphatase 2 γ and type II cGMP-dependent protein kinase are localized to mammalian cilia.

A subset of membrane proteins appear to be anchored to the axoneme

Peptides derived from several proteins predicted to be membrane proteins, including the three PMCA and the polycystin 2 homologue, were more abundant in the axonemal fractions than in the membrane + matrix fraction. It is unlikely that these proteins were collected with the axonemes simply due to an association with membrane that resisted the detergent extraction, because (a) no membrane was observed in the axonemal fractions by electron microscopy, and (b) peptides from other predicted membrane proteins, such as the InsP3 receptor and 14 out of 18 of the proteins with myristoylation domains, were found almost exclusively in the membrane + matrix fraction. This would not be expected if there were undetected membrane contaminating our axonemal fractions. More likely, membrane proteins whose peptides were most abundant in the axonemal fractions are linked to the axoneme in a detergent-resistant manner. This would be consistent with a large body of literature (for review see Dentler, 1990) supporting linkages between ciliary membrane proteins and the axoneme. For example, thin filamentous structures that connect the membrane to the sides of the outer doublet microtubules are commonly observed in electron micrographs of thin-sectioned cilia (Dentler, 1990). Longitudinal rows of intramembranous particles have been observed on the faces of freeze-fractured ciliary membranes (Gilula and Satir, 1972; Sattler and Staehelin, 1974; Bergman et al., 1975); the distance between these rows is close to that between adjacent outer doublet microtubules, suggesting that the particles are linked to the underlying doublet microtubules. In the alga *Ochromonas*, the mastigonemes (thin, hair-like structures projecting from the extracellular surface of the flagellar membrane) are connected to the doublet microtubules and remain connected even after removal of the flagellar membrane by detergent

extraction (Markey and Bouck, 1977). Finally, in *Chlamydomonas moewusii*, there is compelling evidence that a surface-exposed flagellar glycoprotein is tightly associated with the axoneme and stays attached to the axoneme after removal of the membrane by detergents (Reinhart and Bloodgood, 1988). Linkage to the axoneme may ensure that these membrane proteins remain correctly distributed along the flagellum.

Comparison with other broad studies of *C. reinhardtii* gene expression

Two recently published studies also examined expression levels of large numbers of *C. reinhardtii* genes in response to deflagellation. Li et al. (2004) used real time PCR to examine 103 genes that had been chosen based on the observation that they were conserved in ciliated species but not in nonciliated species. 33 of these also were examined by us and the results for these are very similar in the two datasets. Stolc et al. (2005) used a gene chip to examine genome-wide expression patterns including all of the genes we identified except FAP41. The Stolc et al. dataset has a high rate of false negatives such that only 54% of genes previously shown to be induced by deflagellation were found to be induced 100% (equivalent to 1 \times induction in our study) or greater. Thus, the lack of induction as measured by the gene chip cannot be interpreted as evidence for nonciliary roles. However, the Stolc et al. (2005) dataset has a very low false positive rate, as indicated by the fact that, within a subset of 45 potential flagellar genes that we examined by real-time PCR analysis and that Stolc et al. reported to be induced at least 100%, 40 were found to be induced at least 1 \times in our analysis. The exceptions were C_1140006, C_200160, and the genes encoding Hsp70, calmodulin, and Ran. Of 220 genes found to be induced at least 100% by Stolc et al. (2005), 155 encode proteins identified in the flagellar proteome by two or more peptides and an additional 18 encode proteins identified by one peptide. The remaining 47 genes may encode flagellar proteins not detected in our analysis, encode proteins that are involved in flagellar regeneration but not incorporated into the flagellum, encode transcripts that are not translated, or be false positives.

Conserved uncharacterized proteins

Remarkably, the flagellar proteome contains 87 proteins that have conserved homologues in humans (BLAST Expect $\leq 1e-10$), but whose homologues have not yet been characterized in any organism. 60 out of 69 (87%) of these genes examined were induced by deflagellation, suggesting that they function primarily in cilia. Only eight of these were previously identified by Ostrowski et al. (2002). This confirms that the protein composition of cilia has not been well characterized, and highlights the need for further research on these proteins.

Conclusions

Our analysis of the *C. reinhardtii* flagellar proteome has identified many proteins of known function not previously ascribed to cilia and flagella, as well as many other proteins whose functions and cellular locations were completely unknown before now. The results indicate that cilia and flagella are far more complex than previously believed. The findings provide a starting point for future

studies to elucidate the roles of these proteins in the assembly and function of these ubiquitous cell organelles, to understand why defects in some of these proteins lead to human diseases, and to clarify the origin of cilia during the evolution of the eukaryotic cell.

Materials and methods

Cell culture and flagellar fractions

C. reinhardtii wild-type strain 137c and mutant strain oda1-1 were grown in liquid culture as previously described (Witman, 1986). In the initial analysis (Fig. 2), flagella were isolated from wild-type cells by the dibucaine method (Witman, 1986), which yields highly intact flagella, and treated with 1% Tergitol type NP-40 (Sigma-Aldrich) in HMDEK (30 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EGTA, 25 mM KCl) at 4°C, which disrupts but does not dissolve the flagellar membrane (Wirschell et al., 2004). The resulting membranes and axonemes were collected by centrifugation (Witman, 1986); the soluble matrix fraction was discarded. In subsequent analyses (Fig. 2), flagella were isolated from cells of the outer armless strain oda1-1 (Kamiya, 1988; Takada et al., 2002) to reduce the protein complexity of the starting preparation, and then demembrated by treatment with 1% Nonidet NP-40 (Calbiochem) in HMDEK at 4°C to dissolve the flagellar membrane and release the matrix proteins. The "membrane + matrix" fraction was then separated from the axonemes by centrifugation and collected (Witman, 1986). The axonemes were extracted with 0.6 M KCl in HMDEK to solubilize inner arm and many other proteins, and the mixture centrifuged to yield supernatant ("KCl extract") and pellet ("extracted axonemes") fractions (King et al., 1986). Proteins of all four fractions (the wild-type Tergitol-insoluble "membrane + axonemes" fraction, and the mutant "membrane + matrix", "KCl extract", and "extracted axonemes") were then separated by one-dimensional SDS-PAGE using 5–15% polyacrylamide gels (King et al., 1986). The gels were stained with Coomassie blue.

Preparation of protein digests and MS

The entire gel lane for each fraction was cut into 33 slices (Tergitol-insoluble membrane + axonemes fraction) or 41–45 slices (oda1 fractions), and proteins were digested "in gel" according to established methods (Lahm and Langen, 2000). Eluted peptides were separated on a LC Packings Ultimate Nanoflow HPLC system as follows: 10 µl of the peptide digest solution (approximately one half of the total digest) was manually injected onto a micro trap column (precolumn cartridge 0.3 mm × 5 mm C18PM; LC Packings) and the trap column was manually washed with 10 µl of 0.1% formic acid before switching in line with the reverse phase separating column (100 µm × 15 cm C18 PepMap; LC Packings). A gradient was developed from 100% solvent A (0.1% formic acid) to 60% solvent B (0.1% formic acid in acetonitrile/water 70:30) in 40 min at a flow rate of 500 nl/min. The outlet of the column was connected to an electrospray needle (20 µm taper tip; New Objective, Inc.). Electrospray MS was performed on a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron Corp.). Data-dependent acquisitions were set up according to a triple play experiment program where full MS scans from 400 D-2000 D were on going until an MS signal grew above a specified threshold at which time a high-resolution scan (Zoom Scan) was performed to determine monoisotopic mass and charge state followed by a single MS-MS scan. Dynamic exclusion was applied to prevent repeat scans of the same peptide masses. The raw data files were converted into mass peak lists using the LCQ_DTA program and then searched against both the Joint Genome Institute version 2 draft assembly of the *C. reinhardtii* genome translated in all six frames and the predicted protein models derived from this assembly (<http://genome.jgi-psf.org/chlr2/chlr2.home.html>). Searches were performed using the Mascot search engine (Matrix Science Ltd.) with 1D mass tolerances for both the parent and fragment masses.

Peptides with Mascot scores >40 were compared to the genome assembly by BLAST to identify the models from which they were derived. In most cases, the peptides could be unambiguously assigned to one model. However, in some cases, such as the tubulins, it was not possible to identify the specific member of the gene family from which the peptide was derived. In other cases, the peptides did not match any predicted model but grouped tightly together, suggesting that this region of the genome encodes an expressed protein that was not predicted (e.g., FAP41). In still other cases, the peptides mapped just outside of predicted genes; if EST data suggested that the gene model should be extended to include the peptide, we associated this peptide with the model.

The peptide sequences of the gene models were compared to the NCBI protein database with BLAST to identify the most closely related proteins in all species and to the predicted proteomes of humans (NCBI build 33) and *Arabidopsis* (Ath1_pep_20030417.z) to determine whether the proteins were conserved in these two species. The chromosomal map position of the best human match was extracted from the NCBI Gene database. The proteins were also put through Interpro domain analysis (Mulder et al., 2005), the multicoil coiled-coil predictor (Wolf et al., 1997), the TMHMM membrane-spanning helix predictor (Krogh et al., 2001), and the MYR myristoylation predictor (Maurer-Stroh et al., 2004). Cluster analysis was performed with Expander (Sharan et al., 2003). The results of these analyses are available at our web site (<http://labs.umassmed.edu/chlamyfp/index.php>).

Real time PCR analysis of gene induction

To measure induction of gene expression by deflagellation, *C. reinhardtii* 137c cells were deflagellated by pH shock, after which the cells synchronously regenerate their flagella, and RNA was isolated 30 min later from the deflagellated cells and from untreated control cells. The RNA was reverse transcribed using Clontech PowerScript Reverse Transcriptase (BD Biosciences) and the relative amount of specific messages in the two samples was determined by real-time PCR using pairs of primers that spanned introns and SYBR green to monitor amplification. The relative amount of the noninduced G protein β subunit (Schloss, 1990) was measured in each trial and used to correct for slight differences in amount of mRNA in each sample. In our terminology, "0× induction," "1× induction" and "2× induction" indicate no change, a doubling, or a tripling of transcript levels, respectively. Three independent sets of RNA were isolated and analyzed. Primer pairs that spanned introns were picked using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

On-line supplemental material

Table S1 shows flagellar proteins identified by two or more peptides. Table S2 shows flagellar proteins identified by a single peptide. Table S3 lists known *C. reinhardtii* flagellar proteins. Figure S1 depicts SDS-polyacrylamide gels used for proteomic analysis. Online supplemental materials are available at <http://www.jcb.org/cgi/content/full/jcb.200504008/DC1>.

We thank Drs. D. Cole, H. Qin, J. Rosenbaum, W. Sale, and P. Yang for providing unpublished sequences, D. Cochran for technical assistance, Dr. S. King for assistance with gel electrophoresis, Dr. S. Maurer-Stroh for the myristoylation predictions, and Drs. S. Merchant, M. Spalding, and O. Vallon for insightful comments.

This work was supported by grants from the National Institutes of Health (NIH; GM-60992 to G.J. Pazour and GM-30626 to G.B. Witman), by a Worcester Foundation for Biomedical Research Foundation Scholar Award (to G.J. Pazour), and by the Robert W. Booth Fund at the Greater Worcester Community Foundation (to G.B. Witman). The University of Massachusetts Medical School (UMMS) Proteomic Mass Spectrometry Lab is partially supported by NIH DK32520 to the UMMS DERC.

Submitted: 1 April 2005

Accepted: 17 May 2005

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